Cholesteryl Ester Hydroperoxide Lability Is a Key Feature of the Oxidative Susceptibility of Small, Dense LDL

Laurent Chancharme, Patrice Théron, Fabienne Nigon, Sylvie Lepage, Martine Couturier, M. John Chapman

Abstract—Abundant evidence has been provided to substantiate the elevated cardiovascular risk associated with small, dense, low density lipoprotein (LDL) particles. The diminished resistance of dense LDL to oxidative stress in both normolipidemic and dyslipidemic subjects is established; nonetheless, the molecular basis of this phenomenon remains indeterminate. We have defined the primary molecular targets of lipid hydroperoxide formation in light, intermediate, and dense subclasses of LDL after copper-mediated oxidation and have compared the relative stabilities of the hydroperoxide derivatives of phospholipids and cholesteryl esters (CEs) as a function of the time course of oxidation. LDL subclasses (LDL1 through LDL5) were isolated from normolipidemic plasma by isopycnic density gradient ultracentrifugation, and their content of polyunsaturated molecular species of phosphatidylcholine (PC) and CE and of lipophilic antioxidants was quantified by reverse-phase high-performance liquid chromatography. The molar ratio of the particle content of polyunsaturated CE and PC species containing linoleate or arachidonate relative to α-tocopherol or β-carotene did not differ significantly between LDL subclasses. Nonetheless, dense LDL contained significantly less polyunsaturated CE species (400 mol per particle) compared with LDL1 through LDL4 (range, ~680 to 490 mol per particle). Although the formation of PC-derived hydroperoxides did not vary significantly between LDL subclasses as a function of the time course of copper-mediated oxidation, the abundance of the C18:2 and C20:4 CE hydroperoxides was uniquely deficient in dense LDL (23 and 0.6 mol per particle, respectively, in LDL5; 47 to 58 and 1.9 to 2.3 mol per particle, respectively, in other LDL subclasses) at propagation half-time. When expressed as a lability ratio (mol hydroperoxides formed relative to each 100 mol of substrate consumed) at half-time, the oxidative lability of CE hydroperoxides in dense LDL was significantly elevated (lability ratio <25:100) relative to that in lighter, larger LDL particle subclasses (lability ratio >40:100) throughout the oxidative time course. We conclude that the elevated lability of CE hydroperoxides in dense LDL underlies the diminished oxidative resistance of these particles. Moreover, this phenomenon appears to result not only from the significantly elevated PC to free cholesterol ratio (1.54:1) in dense LDL particles (1.15:1 to 1.25:1 for other LDL subclasses) but also from their unique structural features, including a distinct apoB100 conformation, which may facilitate covalent bond formation between oxidized CE and apoB100.

Key Words: LDL subclasses ■ reversed-phase high-performance liquid chromatography ■ lipophilic antioxidants ■ cholesteryl ester hydroperoxides ■ polyunsaturated fatty acids

The formation of atherosclerotic plaques in the major arteries underlies the clinical manifestation of the commonest forms of cardiovascular disease, ie, myocardial infarction, angina, and stroke. Extensive chemical analyses have now established that the major lipid components of these plaques are cholesterol, its esters, and a complex series of oxidized derivatives.1–3 Circulating LDL, in which more than half of the particle mass is accounted for by cholesteryl oleate and free cholesterol (FC), represents the major source of such plaque lipids.4 The observation that elevated plasma levels of LDL represent a major risk factor for the premature development of coronary artery disease (CAD) is entirely consistent with the key role of these cholesterol-rich particles in the formation of atherosclerotic lesions.5 Nonetheless, similar plasma levels of LDL may confer dramatically different cardiovascular risk.6 This situation reflects the variable contribution of distinct subpopulations of LDL particles of elevated atherogenicity to the total LDL fraction.6–7 Indeed, attention has been drawn only recently to the qualitative heterogeneity of plasma LDL; nonetheless, abundant evidence has been provided to substantiate the abnormal quality of LDL particles in CAD patients, which are characterized by a predominance of small, dense LDL.8–14

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As a consequence of their variable contents of lipids, plasma LDL particles are distributed as a continuum over the density range 1.018 to 1.065 g/mL. Multiple subpopulations of LDL particles, which differ in both their physicochemical properties and biological functions, may be found within this continuum. For practical purposes, LDL particles may be grouped on a structural and metabolic basis into a minimum of 3 major subclasses: light, large LDL (d=1.018 to 1.030 g/mL); intermediate LDL (d=1.030 to 1.040 g/mL); and small, dense LDL (d=1.040 to 1.065 g/mL). All LDL particles contain a single copy of apoB100; these particles vary markedly, however, in their absolute lipid content, which may represent from ~70% in small, dense LDL to 80% by weight in light, large LDL. In addition, LDL particles also vary in the relative proportions of their major lipid components (eg, cholesteryl esters [CEs], free cholesterol [FC], triglycerides, and phospholipids [PLs]) and in their contents of minor lipids (eg, gangliosides, plasmalogens) and fat-soluble vitamins (eg, ubiquinol-10, oxygenated carotenoids, and vitamin E). Lipid radicals, which may represent from 70% in small, dense LDL to 80% by weight in light, large LDL, are retained on high-affinity binding to extracellular matrix components. Indeed, matrix binding appears to facilitate oxidative modification, thereby exacerbating the diminished resistance to oxidative stress characteristic of small, dense LDL. In this way, the catabolism of modified forms of dense LDL by atherogenic pathways, such as that represented by the scavenger receptor pathway of monocyte-derived macrophages, is potentiated.

Several hypotheses have been formulated to account for the elevated susceptibility of dense LDL to copper-mediated oxidation. Prominent among them are the following: a deficient content of lipophilic antioxidants (ubiquinol-10, vitamin E, and oxygenated carotenoids), an elevated proportion of polyunsaturated fatty acids (PUFAs), and altered properties of the surface lipid monolayer, which includes a reduced content of FC. Moreover, the latter property has been proposed to confer diminished protection against free-radical attack.

The molecular mechanisms inherent to the elevated susceptibility of dense LDL particles to oxidative stress remain, however, to be defined. According to Esterbauer et al, the formation of lipid hydroperoxides (LOOHs) is a key step in the Cu2+-stimulated oxidation of LDL. These initial reaction products result principally from attack by alkyl and peroxyl radicals at double bonds in the PUFAs of LDL PLs and CE.

In the presence of a transition metal ion such as copper, LOOHs constitute a continuous source of new, lipid alkoxyl and lipid peroxy radicals that can initiate further chain reactions, thereby acting as amplifiers of lipid peroxidation.

In addition, the decomposition of such lipid radicals leads not only to production of reactive aldehydes but also to cleavage of apoB100. Clearly then, the lability of hydroperoxides of PLs and CE in LDL particles is a critical component of the oxidative susceptibility of LDL particles. The objectives of this study were therefore (1) to define the primary molecular targets of LOOH formation in the light, intermediate, and dense LDL subspecies of normolipidemic subjects on copper-mediated oxidation and (2) to evaluate the lability of such LOOHs in atherogenic, dense LDL particles and compare it to that of LOOH species in the light and intermediate LDL subclasses. By determination of hydroperoxide content relative to the oxidative consumption of PUFAs containing PLs and CE, we demonstrate that the relative degree of lability of CE hydroperoxides (CLOOHs) in human LDL subspecies is markedly elevated in small, dense LDL during copper-mediated oxidation.

**Methods**

**Reagents and Chemicals**

High-performance liquid chromatography (HPLC)–grade solvents and analytical-grade chemicals were from Carlo Erba or Merck. Microperoxidase (MP11), isoluminol (6-amino-2,3-dihydro-1,4- phthalalazinedione), and 15-hydroperoxyecosatetraenoic acid (15(s)HPETE) were from Sigma Chemical Co.

**Blood Samples**

Venous blood was collected into sterile, evacuated tubes (Vacutainer) containing K2EDTA (final concentration, 1 mg/mL; reference 367655, Becton Dickinson) from healthy, normolipidemic male subjects after an overnight fast. None of our volunteers was receiving antioxidant vitamin supplementation or drugs known to affect lipoprotein metabolism; subjects were nonsmokers and either abstainers or only moderate alcohol consumers. Plasma was immediately separated by low-speed centrifugation (1000g for 20 minutes) at 4°C; gentamicin (final concentration, 50 μg/mL; Schering-Plough), EDTA (final concentration, 0.1 mg/mL), and Pefabloc (final concentration, 0.35 mg/mL; Pentapharm) were then added to inhibit microbial growth, metal cation–catalyzed peroxidative degradation of lipoproteins, and serine protease activity, respectively.

**Isolation of Plasma LDL Subfractions**

Lipoproteins were fractionated by isopycnic density gradient ultracentrifugation by the method described by Chapman et al with slight modifications. In brief, plasma density was first increased to 1.21 g/mL by addition of solid KBr (0.322 g/mL plasma). Continuous density gradients were then constructed in Ultraclear tubes (Beckman No. 344059) of a Beckman SW41 swinging-bucket rotor. Salt solutions were made from NaCl and KBr and contained 0.1 mg/mL EDTA and 0.05 mg/mL gentamicin. Densities were verified to the fourth decimal place with a precision densitometer (model DMA 40, Anton Paar) at 15°C. Gradients were centrifuged in the SW41 rotor in a Beckman XL-70 ultracentrifuge for 46 hours at 15°C and 40 000 rpm (56.7X10^6 g, 1 minute). After centrifugation, 2 successive fractions, each 0.4 mL, were removed from the meniscus downward by aspiration with a narrow-bore Pasteur pipette; these fractions corresponded to VLDL (d<1.016 g/mL) and LDL (1.017<d<1.018 g/mL), respectively. The next 4 mL of each gradient (containing LDL and corresponding to an overall density range of 1.018 to 1.065 g/mL) was then fractionated into successive 0.8-mL subfractions by aspiration with a Gilson precision pipette. The 5 subfractions of LDL obtained corresponded to the following density intervals: LDL1, 1.018 to 1.023 g/mL; LDL2, 1.023 to 1.030 g/mL; LDL3, 1.030 to 1.040 g/mL; LDL4, 1.040 to 1.051 g/mL; and LDL5, 1.051 to 1.065 g/mL. Corresponding LDL subfractions in different gradient tubes derived from the same plasma were pooled and maintained at 4°C under argon until analysis. In view of the low concentrations of lipoproteins in subfractions 1 and 2, they were pooled and termed “LDL1+2” or “light LDL” (d=1.018 to 1.030 g/mL); LDL3 corresponds to the “intermediate” subclass, and LDL4 and LDL5 are each considered as dense subclasses (density range, 1.040 to 1.065 g/mL). Analysis of the hydroperoxide content of phosphatidylcholine (PC) and CE of normolipidemic LDL subfractions by HPLC (see below) with chemiluminescence detection was performed to evaluate the possibility that LDL oxidation occurred during the isolation procedure; we failed to detect such components.
Analysis of Plasma Lipids, Lipoprotein Lipids, and Apoproteins

These analyses were performed by a series of procedures described in detail earlier. In brief, total plasma cholesterol and triglyceride concentrations were determined by a modification of the Liebermann-Burchard reaction and by the method of Kessler and Lederer after zeolite extraction, respectively. “Seronorm lipid” (Neygaard AS) was used as the working standard for both assays. Plasma HDL cholesterol was estimated by the method of Allain et al using an enzymatic kit (Bioroll). LDL cholesterol was calculated using the Friedewald formula. Plasma apoB, apoA1, and lipoprotein(a) levels were measured by immunological assays as described earlier and based on laser immunophenophotometry (Immuno AG).

Chemical Analysis of Lipoprotein Subfractions

Chemical analyses of LDL subfractions were performed by the series of procedures that we originally described, including protein quantification by the bicinchoninic acid protein assay (Pierce), measurement of FC and esterified cholesterol by the method of Roeschau et al, PL estimation by the procedure of Takayama et al, and triglyceride measurement by the technique of Biggs et al. Enzymatic assay kits (Boehringer) were used for FC and total cholesterol estimations; CE was calculated as (total cholesterol - FC)/1.67 as indicated earlier. PL and triglyceride estimations were performed with enzymatic kits (BioMerieux). Analyses were performed in triplicate, and the technical errors in these analyses were similar to those determined earlier. Calibration curves for lipoprotein assays were established with purified standards in each case. The total LDL mass in each LDL subfraction was calculated as the sum of the concentrations of the lipid and protein components and allowed determination of the percent chemical composition.

Determination of Antioxidant Content

The particle content of α-tocopherol and carotenoids (α- and β-carotene, lutein, lycopene, and cryptoxanthin) were determined in each LDL subfraction by reverse-phase HPLC after protein precipitation by ethanol and extraction of the lipophilic antioxidants with hexane. A C18 5-µm Hypersil ODS column (25 cm × 4.6 mm internal diameter; reference H225, Shandon) and a Chromatofield pump were used. The mobile phase, consisting of a mixture of acetonitrile, methylene chloride, and methanol (70:20:10, vol/vol/vol), was pumped at a flow rate of 0.7 mL/min. The working standard for the α-tocopherol assay was α-tocopherol (Eastman Kodak, reference 6340); D-α-tocopherol acetate (Eastman Kodak, reference 6692) was used as the internal standard. Standards for β-carotene, α-carotene, lutein, and lycopene were Sigma products (respectively, references C0126, C0251, N6250, and L9879). α-Tocopherol and carotenoids were detected at 292 and 450 nm, respectively (Shimadzu UV-visible spectrophotometric detector).

Determination of the Oxidative Susceptibility of LDL Subfractions

The susceptibility of LDL subfractions to in vitro copper-mediated oxidation was assessed by the technique described by Esterbauer et al. Each LDL subfraction was first dialyzed in Spectrapor membrane tubing (relative molecular mass cutoff, 12 000 to 14 000) for 36 hours at 4°C against 3 changes of 5 L of 0.01 mol/L phosphate buffer (pH 7.4) containing 0.16 mol/L NaCl (dialysis buffer). EDTA-free LDL subfractions were then diluted in dialysis buffer to a final concentration of 250 μg/mL LDL (as total mass) per milliliter, and oxidation was initiated by addition of aqueous CuCl2, solutions to a final concentration of 1.6 μmol/L. The time course of LDL oxidation was monitored continuously at 234 nm and at 37°C on a recording spectrophotometer (Kontron). The conjugated dienes formed during LDL oxidation produce an absorption spectrum with a distinct peak at 234 nm; the initial absorbance at 234 nm was taken as the baseline, and absorbance was recorded every 3 minutes for 4 hours. The absorbance curve at 234 nm was divided into 3 phases: a lag phase, a propagation phase, and a decomposition phase. From the absorbance profile for each LDL subfraction, 3 characteristic time points (expressed in minutes) can be determined that describe the oxidative behavior of the LDL preparation. The first time point, the lag time (Tlag), was defined as the intersection of the baseline with the tangent to the slope of the absorbance curve during the propagation phase. Second, the maximum time (Tmax) was defined as the time at the end of the propagation phase when diene production reached its maximal value. The third time is the propagation half-time (T1/2) and corresponds to the midpoint of the propagation phase.

In addition to these parameters, we equally determined the following: (1) the maximal amount of dienes produced, which was expressed as mole per mole LDL, and (2) the maximal rate of diene formation, which was calculated from the slope of the absorbance curve during the propagation phase and expressed as moles of dienes produced per minute per mole of LDL (see Table 2).

At each of the 3 oxidation time points (see above), 1 mL of the oxidation mixture of each LDL subfraction was withdrawn, and a 0.025% (wt/vol) alcoholic solution of BHT (Farmitalia Carlo Erba SpA) was added to inhibit lipoperoxidation. Samples were then stored at −80°C until analysis by HPLC.

Lipid Extraction and Separation of PLs and CEs

Lipids were extracted with methanol/hexane (4:10, vol/vol) from aliquots of LDL subfractions corresponding to each of the 3 time points of copper oxidation defined above. The mixture was partitioned between the hexane-water (upper phase, containing CEs) and the methanol/water (lower phase containing PLs); these layers were separated by centrifugation at 1500g for 5 minutes. The 2 layers were collected and evaporated to dryness under a nitrogen stream; the dried lipid residues were then redissolved in methanol and injected into the HPLC system (see below).

Chromatographic Analysis of Molecular Species of PLs and CEs

The HPLC equipment included an automatic injector with a 200-μL sample loop, a UV–visible light detector (Thermo Separation Products), and a fluorometer equipped with a 5-μL flow cell (Spectroflow 980 fluorescence detector, Applied Biosystems). PL subspecies were separated by using 2 serial analytical columns: a 250 × 4.6-mm C18 Kromasil 5 μm (Touzart et Matignon). The mobile phase consisted of a solution containing 6% of 10 mmol/L ammonium acetate (pH 5) and 94% methanol (flow rate, 1.5 mL/min). CE separation was performed with a 250 × 4.6-mm C18 Spherisorb column; the mobile phase was methanol. Molecular species of PC and CEs were detected at 205 nm, and the eluate was then mixed with the chemiluminescence reagent prepared as described by Yamamoto et al with slight modifications: isoluminol (55 mg) was dissolved in 0.1 mol/L borate buffer (pH 9.2), and 10 mg/L microperoxidase was added. The chemiluminescence reagent was subsequently passed (flow rate, 1 mL/min) through a fluorometer used as a photon detector with the excitation source extinguished. This methodology had been validated earlier by Therond et al. Identification of Molecular Species of PLs and CEs and Their Corresponding Hydroperoxides

On the 2 chromatographic profiles, each peak corresponding to an esterified PC or CE was identified by its retention time relative to commercial standards (Nu-Chek-Prep for esterified PC and Sigma for esterified CE). The concentration of each component was determined by comparing the surface of the peaks to that of standards. Hydroperoxides from each molecular species of PC (linoleate, arachidonate, and docosahexaenoate) or CE (linoleate and arachidonate) were prepared using lipoxygenase (type I-B, Sigma). In brief, various molecular species of PC and CE were dissolved in 1 mL of 0.1 mol/L borate buffer, pH 9.2, containing 10 mmol/L deoxycholic acid. One hundred ten microliters of a solution of lipoxigenase (prepared in 0.1 mol/L borate buffer, pH 9.2; 500 μg/mL) was then added, and the mixture was incubated with gentle shaking at 30°C for 45 minutes. The reaction was stopped by adding 100 μL of 1 mol/L citric acid. Hydroperoxides from PC and CEs were extracted with methanol/hexane as described above for LDL. The various fractions (hexane for CE/COOHs and methanol for PC/COOHs) were evaporated to dryness, redissolved in methanol, and then injected into the HPLC system (see conditions above). The products were detected by chemiluminescence and identified by their
TABLE 1. Chemical Composition of LDL Subfractions in Normolipidemic Subjects

<table>
<thead>
<tr>
<th>Component, mol/mol LDL</th>
<th>LDL1 + 2 (d=1.018–1.030 g/mL)</th>
<th>LDL3 (d=1.030–1.040 g/mL)</th>
<th>LDL4 (d=1.040–1.051 g/mL)</th>
<th>LDL5 (d=1.051–1.065 g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>1914±188*§</td>
<td>1798±66*§</td>
<td>1486±88*</td>
<td>1099±151</td>
</tr>
<tr>
<td>FC</td>
<td>798±45*§§</td>
<td>677±46*§§</td>
<td>529±82*</td>
<td>375±50</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>315±11*§</td>
<td>141±38#</td>
<td>136±39#</td>
<td>117±39#</td>
</tr>
<tr>
<td>PL</td>
<td>918±59*</td>
<td>780±41#§</td>
<td>656±49#§</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>1.02±0.10</td>
<td>1.00±0.11</td>
<td>1.07±0.18</td>
<td>1.20±0.24</td>
</tr>
<tr>
<td>PL/FC</td>
<td>1.15±0.10‡</td>
<td>1.16±0.06‡</td>
<td>1.26±0.16</td>
<td>1.54±0.39</td>
</tr>
<tr>
<td>Antioxidant content, mol/mol LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>10.6±2.0</td>
<td>8.4±2.0</td>
<td>6.7±1.9#</td>
<td>6.4±1.1#</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.33±0.10‡</td>
<td>0.33±0.11‡</td>
<td>0.24±0.06</td>
<td>0.20±0.04</td>
</tr>
<tr>
<td>PUFAs: α-tocopherol ratio, mol/mol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PUFAs in CE and PL</td>
<td>84±16</td>
<td>101±27</td>
<td>100±35</td>
<td>89±19</td>
</tr>
<tr>
<td>CE containing PUFAs</td>
<td>61±14</td>
<td>74±22</td>
<td>73±25</td>
<td>60±13</td>
</tr>
<tr>
<td>PL containing PUFAs</td>
<td>23±5</td>
<td>26±7</td>
<td>27±10</td>
<td>28±9</td>
</tr>
<tr>
<td>PUFAs: β-carotene ratio, mol/mol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PUFAs in CE and PL</td>
<td>2891±961</td>
<td>2998±1421</td>
<td>3191±1437</td>
<td>3182±1063</td>
</tr>
<tr>
<td>CE containing PUFAs</td>
<td>2120±788</td>
<td>2227±1140</td>
<td>2356±1139</td>
<td>2185±885</td>
</tr>
<tr>
<td>PL containing PUFAs</td>
<td>807±257</td>
<td>765±274</td>
<td>833±301</td>
<td>986±221</td>
</tr>
</tbody>
</table>

For chemical composition analysis, values are means±SD of triplicate determinations of each component in 8 separate preparations. Analyses were performed as described in Methods. Molecular weights employed for the calculations are as follows: 3.04×10^6, 2.62×10^6, 2.33×10^6, and 2.00×10^6 D for LDL1 + 2, LDL3, LDL4, and LDL5, respectively. Molecular weights of CEs, FC, triglyceride, PL, and protein are, respectively, 650, 387, 850, 750, and 513×10^6 D. α-Tocopherol, β-carotene, and PUFAs were analyzed by HPLC (see Methods). Values are mean±SD for 6 separate preparations. PUFAs included linoleic acid (C18:2), arachidonic acid (C20:4), and docosahexaenoic acid (C22:6).

*P<0.0001, †P<0.002, and ‡P<0.005 statistically different from LDL5.
¶P<0.0001 statistically different from LDL4.
§P=0.0003, †P<0.001 statistically different from LDL3.
#P<0.0001 statistically different from LDL1 + 2.

Statistical Analysis

All results are presented as mean±SD. The differences between the mean values for the chemical composition, T lag , the oxidative modification of PL and CE species containing PUFAs, and the hydroperoxide content of PL and CE components of LDL subfractions were analyzed by ANOVA. When the F statistic was significant (P<0.05), the Bonferroni test was used to determine differences between LDL subfractions. Statistical analyses were performed with a Stat-View 4.05 program (Abacus Concepts).

Results

Mass Distribution of LDL Subfractions

In this series of normolipidemic subjects, the mass distribution of LDL subfractions as a function of density was characterized by a predominance of the intermediate subclass (LDL3, d=1.030 to 1.040; 102.5±51.8 mg/dL; 46% of the total mass of plasma LDL); by contrast, the levels of dense LDL (LDL4, 54.1±17.8 mg/dL and LDL5, 24.2±6.4 mg/dL) represented 35% of the total mass of plasma LDL. The mean concentration of light, large particles in the LDL1 + 2 subclasses (d=1.018 to 1.030; 44.1±20.3 mg/dL) was less than that of LDL4. Such a quasi-symmetrical LDL mass profile is typical of normocholesterolemic subjects and confirms previous studies.26

Chemical Composition of LDL Subfractions

The chemical compositions of the LDL1 + 2, LDL3, LDL4, and LDL5 subfractions are presented in Table 1. Data are expressed as the mean number of molecules of each chemical component per particle species and were calculated from the respective molecular weights determined earlier for corresponding subfractions.16 It is evident that CE molecules constitute the major component of all LDL subfractions (range, 1099 to 1914 per particle). Furthermore, the number of CE molecules per particle subspecies decreased significantly with an increase in density. The FC and PL components showed a similar trend, whereas the number of molecules of TG diminished by one third from LDL1 to LDL5. Such a quasi-symmetrical LDL mass profile is typical of normocholesterolemic subjects and confirms previous studies.26
number of β-carotene molecules per LDL also decreased progressively with hydrated density. Light and intermediate LDL contained significantly more β-carotene than did denser particles. The study of other lipophilic carotenoid antioxidants did not reveal any significant differences between LDL subspecies (data not shown). These data confirm those reported earlier by our laboratory in a separate series of studies did not reveal any significant differences between LDL particles. The study of other lipophilic carotenoid antioxidants did not reveal any significant differences between LDL subspecies (data not shown). These data confirm those reported earlier by our laboratory in a separate series of studies.

Esterified PUFA Content of PLs and CEs in LDL Subfractions

The content of linoleic (C18:2) and arachidonic (C20:4) acids esterified as components of either PC or CE in each LDL subspecies is shown in Figure 1. The docosahexaenoic acid (C22:6) content of PL species was also measured, but as a result of trace amounts of this PUFA, PLs containing C20:4 and C22:6 were combined. PUFAs were primarily esterified in the form of CEs in all LDL subspecies (400 to 680 molecules per particle; Figure 1B) compared with PLs (180 to 250 molecules per particle; Figure 1A). Linoleate was more abundant than arachidonate in both PLs (≈3-fold more) and CEs (≈13-fold more; data not shown). Because of their abundance, linoleate-containing lipid esters therefore represent the major substrate for lipid peroxidation in all LDL subspecies on a quantitative basis.

Characteristics of Copper-Mediated Oxidation in LDL Subfractions

As reported earlier,26 light, large LDL (LDL1+2) and intermediate LDL (LDL3) displayed a longer Tlag than did dense LDL (P<0.05 for LDL1+2; Table 2), showing them to be more resistant to copper-mediated oxidative stress than denser subspecies. Furthermore, T1/2 and Tmax values diminished progressively from light to intermediate to dense LDL, in parallel with the reduction in Tlag. Moreover, as could be predicted from the data in Table 1, maximal diene production diminished progressively from light LDL to dense LDL subspecies (P<0.0001; Table 2); a similar overall pattern was seen for the maximal rates of diene formation across the LDL subspecies (P<0.0005 for dense LDL versus LDL1+2). However, the enhanced resistance of LDL1+2 in the present studies was not accounted for on the basis of particle antioxidant content. Indeed, when the molar amounts of the primary targets of lipid oxidation, ie, PUFA-containing PC and CE species, was expressed relative to molar particle contents of vitamin E, this molar ratio did not differ significantly between light, intermediate, and dense LDL subfractions (overall range, 61:1 to 74:1 and 23:1 to 28:1 for PUFA-containing CEs and PLs, respectively, relative to α-tocopherol; Table 1). Therefore, each molecule of α-tocopherol protects a similar number of PUFAs esterified in CEs or PC in each LDL subfraction. Nonetheless, as shown in Table 1 and in earlier studies,19 absolute levels of α-tocopherol per LDL particle were significantly lower in dense LDL (LDL4 and 5; 6.5 molecules per particle) than in lighter subspecies (8.4 to 10.6 molecules per particle; P<0.0001).

Oxidative Modification of PUFAs and LOOH Formation

The oxidative degradation of esterified PUFAs in native LDL subspecies was first determined and revealed that 20:4 esters in both CEs and PC were degraded more rapidly and to a greater degree than those containing 18:2 (data not shown). For example, at Tmax of the time course of copper-mediated oxidation, ≈70% of 20:4 esters were no longer present in LDL1+2 (P<0.01). Indeed, LOOH species from polyunsaturated PC and CEs at Tlag, T1/2, and Tmax (with the exception of PC at Tlag). Indeed, LOOH species derived from PC18:2 were 5-fold or more abundant than those derived from PC20:4 (Figure 2A and 2B), whereas those derived from CE18:2 were 20-fold (or more) abundant than those formed.
from CE20:4 (Figure 2C and 2D). Significant differences were equally detected in the amounts of LOOHs formed as a function of LDL subspecies (Figure 2). Thus, the pattern of hydroperoxide formation was correlated positively with the initial particle content of molecular CE and PC species containing PUFAs (Figure 2 and Table 1). However, the abundance of CEOOHs derived from either CE18:2 or CE20:4 was significantly less in dense LDL (LDL5) than in lighter subspecies (LDL1+2, LDL3, and LDL4) at both T_{1/2} and T_{max}.

Because individual LDL subspecies are characterized by distinct contents of PC and CE molecules on a particle basis, we next expressed these data as a function of the initial, native lipid content (Figure 3), ie, as a ratio of the number of molecules of LOOHs formed from a defined molecular PUFA-containing species of PC and CE relative to the

<table>
<thead>
<tr>
<th>LDL1+2</th>
<th>LDL3</th>
<th>LDL4</th>
<th>LDL5</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_{lag}, min</td>
<td>50.8±9.0</td>
<td>41.5±11.6</td>
<td>38.5±10.4</td>
</tr>
<tr>
<td>T_{1/2}, min</td>
<td>77.8±13.4</td>
<td>69.1±14.5</td>
<td>64.8±14.3</td>
</tr>
<tr>
<td>T_{max}, min</td>
<td>107.3±17.7</td>
<td>98.8±16.9</td>
<td>91.4±19.9</td>
</tr>
<tr>
<td>Maximal propagation rate, (mol/mol LDL)/min</td>
<td>10.8±2.7</td>
<td>8.4±1.8</td>
<td>8.0±1.9</td>
</tr>
<tr>
<td>Maximal diene production, mol/mol LDL</td>
<td>381±37</td>
<td>319±25†</td>
<td>275±29‡</td>
</tr>
</tbody>
</table>

Aliquots of LDL subfractions (250 μg LDL mass per mL) were oxidized in the presence of 1.6 μmol/L cells for 3 hours at 37°C as described in Methods. The maximal propagation rate of oxidation was calculated from the slope of the absorbance curve during the propagation phase by using the molar absorptivity for conjugated dienes (ε_{234}=29 500 L·mol⁻¹·cm⁻¹). The maximal amount of conjugated dienes formed at T_{max} is shown. Values are mean±SD of duplicate determinations for 7 separate preparations.

*P<0.005, †P<0.0001, ‡P<0.001, and §P<0.0005 vs LDL1+2. |

P<0.0001 and ¶P<0.0005 vs LDL5.

Figure 2. Formation of LOOHs derived from PC species containing linoleate (A, PCOOH18:2), arachidonate, or docosahexaenoate (B, PCOOH20:4 and 22:6) and from cholesteryl linoleate (C, CEOOH18:2) and cholesteryl arachidonate (D, CEOOH20:4) as a function of the time course of copper-mediated oxidation of LDL subfractions (open box, LDL1+2; light gray box, LDL3; medium gray box, LDL4; and filled box, LDL5). Molecular lipid species were separated by HPLC and detected by chemiluminescence. Results are expressed as the number of hydroperoxide molecules formed per LDL particle (mean±SD, n=5). ★Fraction statistically different from LDL5 (P<0.005).
number of molecules of the same species that had been transformed to an oxidatively modified form. As such, this ratio reflects not only the yield of LOOH species but also their relative lability. When expressed as this ratio, the yield of LOOHs from both PC18:2 and PC20:4 species increased as a function of the progression of oxidative modification in all LDL subfractions (Figure 3A and 3B); no significant differences in the yield of LOOHs were detectable between LDL subclasses for each PUFA-containing PL species. By contrast, the pattern of LOOH formation expressed relative to oxidative substrate consumption was quite distinct for molecular species of CEs (Figure 3C and 3D). Thus, the ratio of LOOH production from CE18:2 relative to consumption increased 2-fold or more at T1/2 and thereafter decreased at T max. Indeed, the ratio attained a peak in LDL3 at T 1/2 (P, 0.001), indicating significant differences between LDL subspecies in the stability of CEOOHs. It is especially relevant that the production to consumption ratio for CEOOH18:2 was lower in dense LDL5 at all phases of oxidation. A similar pattern was observed for the ratio of hydroperoxide content relative to substrate consumption in the case of CE20:4 (Figure 3D), in which maximal LOOH content was observed at both T1/2 and T max, with the exception of LDL5. Indeed, the amounts of LOOHs detected relative to substrate consumed were consistently lower at T1/2 and T max in the densest LDL species, although absolute values of this ratio increased ~3-fold from T lag to T max in this fraction.

When the ratio of CEOOH content to lipid substrate consumption was expressed as a function of the time course of copper oxidation (Figure 4), the aforementioned dissimilarities in the stabilities of LOOH species between LDL subspecies became pronounced. Indeed, not only was significantly less hydroperoxide derived from both the 18:2 and 20:4 esters detected in dense LDL5 at all time points but in addition, the ratio attained a peak earlier for CE18:2-derived hydroperoxides in dense LDL5. Furthermore, the lability ratio was 3-fold to 4-fold greater in LDL3 for both CEOOH18:2 and 20:4 compared with those in LDL5. These data demonstrate the existence of significant differences in LOOH formation and stability between LDL subspecies during copper-mediated oxidation.

**Discussion**

The molecular basis of the diminished oxidative resistance of atherogenic, small, dense LDL remains incompletely understood. It is therefore significant that despite the fact that CE20:4 and CE18:2 represent the major targets of hydroperoxide formation in all LDL subspecies on copper-mediated oxidation, we specifically observed a markedly elevated lability of molecular species of CEOOHs formed within small, dense LDL particles during the propagation phase. These findings could not be accounted for on the basis of either particle contents of PUFAs or vitamin E, because the molar PUFA to α-tocopherol ratio did not vary significantly.
Moreover, the particle content of molecular species of PC containing 18:2 in LDL4 and LDL5 is statistically diminished compared with that of LDL1+2 (P<0.05). By contrast, no differences in particle content of PLs containing highly unsaturated PUFAs (arachidonic and docosahexaenoic acids) were detected between LDL subclasses. A similar tendency was observed with respect to polyunsaturated molecular species of CEs, for which the overall contents were similar in LDL subfractions, with the exception that LDL4 and LDL5 contained less 18:2 than did LDL3 (P < 0.05), whereas LDL5 contained less 20:4 than did LDL1+2 (P < 0.05).

Comparison of the ratio of the particle contents of CE18:2 or CE20:4 to that of PLs containing the same fatty acids did not reveal any dissimilarities between LDL subclasses. However, we detected a tendency toward a lower ratio in dense LDL due to a lower CE18:2 content relative to the other LDL subfractions (2.67±0.72 in LDL5, 3.44±0.69 in LDL4, 3.44±0.75 in LDL3, and 3.42±0.82 in LDL1+2 for CE18:2; 0.65±0.14 in LDL5, 0.80±0.20 in LDL4, 0.84±0.07 in LDL3, and 0.84±0.15 in LDL1+2 for CE20:4). Overall then, these data indicate that dense LDL particles tend to contain fewer polyunsaturated lipid esters per particle than do lighter, larger LDL particles and that this deficiency was particularly marked in molecular species of CEs (Figure 1). Dense LDL particles are therefore not enriched in polyunsaturated CEs and PLs, and it is improbable that this compositional feature could account for their elevated susceptibility to oxidation.

As reported earlier, determination of T₉₀ for conjugated diene formation confirmed that dense LDLs are more susceptible to copper-induced oxidation than are light LDLs (P < 0.05, Table 2). As shown by Esterbauer et al., the oxidation rate of LDL is progressively diminished as a function of increase in its α-tocopherol content. However, the oxidizability of LDL by copper was negatively correlated with LDL α-tocopherol content only when the lipoprotein was enriched in vitro with this antioxidant. By contrast, no significant correlation between vitamin E and any index of LDL oxidation was found in native, nonenriched LDL and indeed, under conditions of low free-radical flux, a prooxidant role of α-tocopherol has been described. Given then that the molar ratio of the particle content of CE or PC species containing PUFAs to α-tocopherol (or β-carotene) was not statistically different between LDL subfractions (Table 1), we conclude that other structural and compositional factors must underlie the increased oxidizability of dense LDL.

Free cholesterol is known to modulate the fluidity of biological membranes. Kontush et al. originally observed that LDL FC content was negatively correlated with LDL oxidizability, whereas its α-tocopherol content was not. Data in Table 1 show that particle contents of FC were significantly reduced in dense LDL5 compared with the larger and lighter LDL subfractions, giving rise to an elevated PL-to-FC ratio. It is established that FC can efficiently decrease the oxidizability of LDL by copper was negatively correlated with LDL oxidizability of LDL by copper was negatively correlated with LDL α-tocopherol content only when the lipoprotein was enriched in vitro with this antioxidant. By contrast, no significant correlation between vitamin E and any index of LDL oxidation was found in native, nonenriched LDL and indeed, under conditions of low free-radical flux, a prooxidant role of α-tocopherol has been described. Given then that the molar ratio of the particle content of CE or PC species containing PUFAs to α-tocopherol (or β-carotene) was not statistically different between LDL subfractions (Table 1), we conclude that other structural and compositional factors must underlie the increased oxidizability of dense LDL.
Present evidence for differences in CEEOH stability among LDL subclasses during copper-mediated oxidation constitutes a major finding. Indeed, Figure 2A and 2B revealed that the formation of PC-derived hydroperoxides did not vary significantly between LDL subspecies as a function of the stage of copper-mediated oxidation. By contrast, formation of CEEOH18:2 and CEEOH20:4 was significantly lower in dense LDL than in other subspecies (Figure 2C and 2D). This diminution was observed both at the midpoint (ie, T_{0.5}) and at the end (T_{max}) of the propagation phase, thereby suggesting that the oxidizability of antioxidant-poor dense LDL is less than that of the other LDL subfractions. However, the ratio of the number of moles of CEEOHs formed from a defined PUFA-containing lipid ester to each 100 moles of the same lipid species present in an oxidatively altered form (defining the lability of CEEOHs) differed significantly among LDL subspecies and was lowest in dense LDL (Figure 3). Thus, the lability of arachidonate hydroperoxides in both CEs and PC was greater than that for linoleate, for all subfractions and for each stage of lipid peroxidation. Second, for a defined molecular species (arachidonate or linoleate), the oxidative lability was markedly lower in CEs relative to PC. These results clearly indicate that hydroperoxide lability in LDL is a function not only of the degree of fatty acid unsaturation but also of the nature of the lipid class itself. 

The finding that the hydroperoxide lability ratio for CEs differs uniquely between dense LDL5 and the larger, lighter LDL1+2 to LDL4). Present evidence for differences in CEEOH stability among LDL subspecies during copper-mediated oxidation constitutes a major finding. Indeed, Figure 2A and 2B revealed that the formation of PC-derived hydroperoxides did not vary significantly between LDL subspecies as a function of the stage of copper-mediated oxidation. By contrast, formation of CEEOH18:2 and CEEOH20:4 was significantly lower in dense LDL than in other subspecies (Figure 2C and 2D). This diminution was observed both at the midpoint (ie, T_{0.5}) and at the end (T_{max}) of the propagation phase, thereby suggesting that the oxidizability of antioxidant-poor dense LDL is less than that of the other LDL subfractions. However, the ratio of the number of moles of CEEOHs formed from a defined PUFA-containing lipid ester to each 100 moles of the same lipid species present in an oxidatively altered form (defining the lability of CEEOHs) differed significantly among LDL subspecies and was lowest in dense LDL (Figure 3). Thus, the lability of arachidonate hydroperoxides in both CEs and PC was greater than that for linoleate, for all subfractions and for each stage of lipid peroxidation. Second, for a defined molecular species (arachidonate or linoleate), the oxidative lability was markedly lower in CEs relative to PC. These results clearly indicate that hydroperoxide lability in LDL is a function not only of the degree of fatty acid unsaturation but also of the nature of the lipid class itself. The third and possibly most significant finding is that CEOOH content between LDL subspecies. Recently, Sattler et al^60 described a CEEOH-reducing activity in LDL that could explain the loss, and thus high lability, of CEEOHs by transformation into hydroxides. Indeed, such activity could predominate in dense LDL and cannot be excluded. Nonetheless, analysis of the HPLC chromatographic elution profile at 205 nm for each LDL subfraction failed to detect preferential enrichment of CE hydroxides in dense LDL (LDL5), thereby excluding this possibility.

One potential explanation for the lower stability of CEEOHs in dense LDL particles is the implication of molecular packing and conformational factors in their reactivity. As the number of molecules of both core and surface constituents decrease in parallel with diminution in particle size and increase in density (Table 1), conformational rearrangement of apoB100 occurs, and indeed it has been shown that apoB100 expands across the core.61–63 Such conformational reorganization of apoB100 in dense LDL appears to underlie the poor binding affinity of these particles for the cellular LDL receptor.21,62 Furthermore, it is well known that structural modification of apoB100 can arise from a concerted reaction between LOOHs and free amino groups in this protein.64,65 Indeed, lipid peroxidation products are known to influence apoB100 conformation.66 We hypothesize that in this way, new domains of apoB100 become accessible and in consequence, new amino acid residues become susceptible to react with CEEOHs in dense LDL.

In conclusion, the present observations suggest that the elevated lability of CEEOHs in dense LDL is a key feature that underlies the diminished resistance of these particles to oxidative stress. This phenomenon appears to result not only from the unique compositional features of dense LDL (elevated PC-to-FC ratio) but also from the distinct conformation of apoB100 in these atherogenic particles.61,63

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References


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Cholesteryl Ester Hydroperoxide Lability Is a Key Feature of the Oxidative Susceptibility of Small, Dense LDL
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